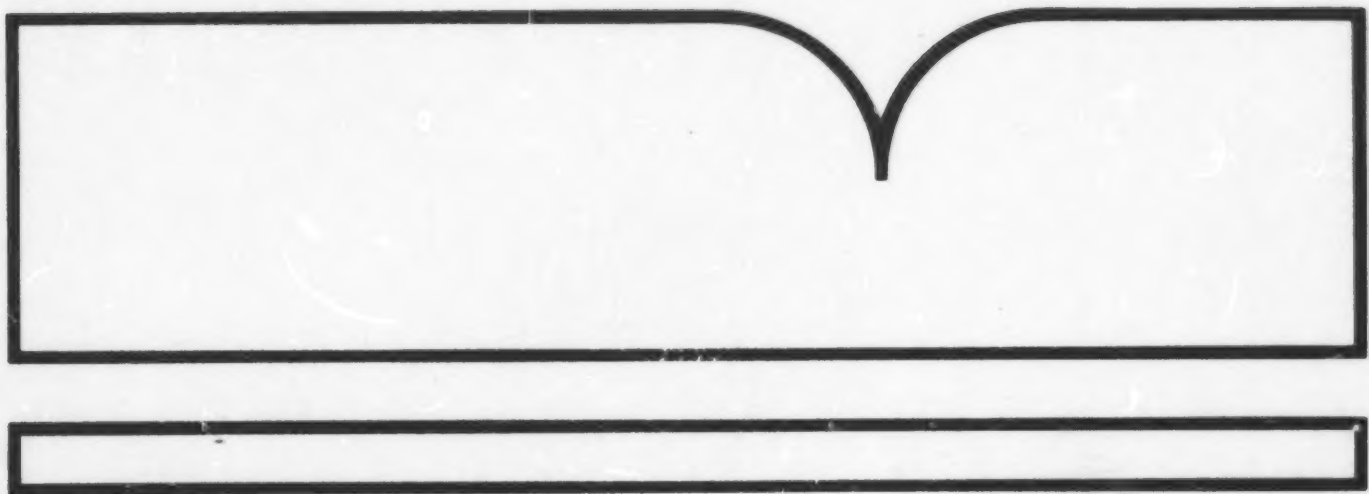


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Membrane-Associated Viral Complexes Observed in Stools and Cell Culture

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Viral complexes observed to be membrane associated rather than clumped by antibody were detected in a rotavirus-containing stool specimen by negative-stain electron microscopy. These "viral packets" were also observed in cell culture fluids after repeated passaging and contained up to 100 virions. Other stool specimens have been observed to contain similar packets of parvovirus-like particles. Such complexes must be expected in fecally contaminated water.

The occurrence and increased resistance of viral aggregates began to be fundamentally addressed in disinfection studies with cell-culture-adapted strains of reovirus and poliovirus (14, 15, 18). Viral aggregates from cell lysates were observed by electron microscopy (EM) to consist either entirely of virions or of virions associated with material presumed to be host cell debris (18). Other *in vitro* studies similarly demonstrated that cell-associated virus was more resistant to disinfection than free virus (8). If naturally occurring, such resistant viral complexes in water may be a public health concern.

Direct evidence that enteric viruses are frequently shed in aggregated states by infected individuals has more recently been demonstrated by EM examination of stools from individuals with gastrointestinal illness (12). It was suggested, however, that the virus particles were likely clumped by the presence of local antibody. The significance of such aggregates remains unclear, as antibody-induced clumping would presumably involve substantial neutralization of virus infectivity.

This report describes stool-shed viral complexes which appear to be intrinsically membrane associated rather than clumped by antibody. These complexes were observed in a rotavirus-containing stool specimen obtained during a waterborne outbreak of gastroenteritis in Colorado (10).

Direct EM examination of this stool specimen was performed by the following procedure. The stool, obviously diarrheal, was thoroughly mixed with a pipette, and enough was removed to provide a moderately turbid suspension in distilled water. One drop of the suspension was then placed on a copper electron microscope grid with carbon substrate and allowed to stand for 1 min. Excess material was removed with a piece of filter paper, and the grid was washed with 1 or 2 drops of distilled water. The grid was then negatively stained with 2% phosphotungstic acid (pH 7). After being dried, the stained grid was examined at 80 kV on a JEOL 100CX electron microscope.

Rotavirus from this stool specimen was successfully propagated in cell cultures after the following preparation. A 1.5-ml portion of the crude stool specimen was added to 8.5 ml of distilled water, and the resulting suspension was centrifuged at low speed ($170 \times g$) for 15 min. The supernatant was retained and further centrifuged at $3,500 \times g$ for 30

min (Beckman SW 50.1 rotor). The supernatant was again retained and passed through 8.0- and 0.45- μ m membrane filters. Before inoculation, this filtered preparation was diluted 1:2 in medium 199 containing 20 μ g of trypsin per ml (bovine pancreas, type 1; Sigma Chemical Co., St. Louis, Mo.) and held at 37°C for 20 min. Aliquots (0.5 ml) of the preparation were then used to inoculate two 25-cm² flasks containing confluent monolayers of RD cells (human rhabdomyosarcoma cells obtained from R. Crowell, Hahnemann Medical College, Philadelphia, Pa.). These flasks had previously been incubated for 4 days with medium 199 (supplemented with 10% fetal calf serum) containing 50 μ g of 5-iododeoxyuridine per ml (3). The monolayers were washed twice with serum-free, 5-iododeoxyuridine-free medium 199 before inoculation. After inoculation, 4.5 ml of serum-free, 5-iododeoxyuridine-free medium 199 containing 10 μ g of trypsin per ml, additional sodium bicarbonate (0.088%), and antibiotics was added to each flask, and the flasks were incubated at 37°C for 3 days. The flasks were then put through three freeze-thaw cycles, and the contents were used to inoculate additional flasks. The culture fluids were also examined by direct EM with the negative-stain technique described above. A total of 19 passages were completed in this manner.

The electron micrographs of membrane-associated virions observed in the rotavirus-containing stool specimen are shown in Fig. 1a through e. Two "viral-packet" complexes (a and b) appeared to contain from 17 to 40 complete double-shelled virions. In their longest dimension, these two packets measured from 0.45 to 0.9 μ m. Individual virions which appeared to be membrane enveloped were also observed (c), as were small groups of membrane-associated virions (d and e). The individual enveloped virions were readily differentiated from both nonenveloped single-shelled and nonenveloped double-shelled particles.

It was not clear what percentage of the total number of virions shed in the stool might have been membrane associated. The most frequently observed virions were double-shelled, dispersed, and not associated with membranous material. Such distinctive individual virions, however, were well visualized by the negative-stain technique and were readily identified. Unfortunately, this was frequently not the case with virions present in the membrane-associated state.

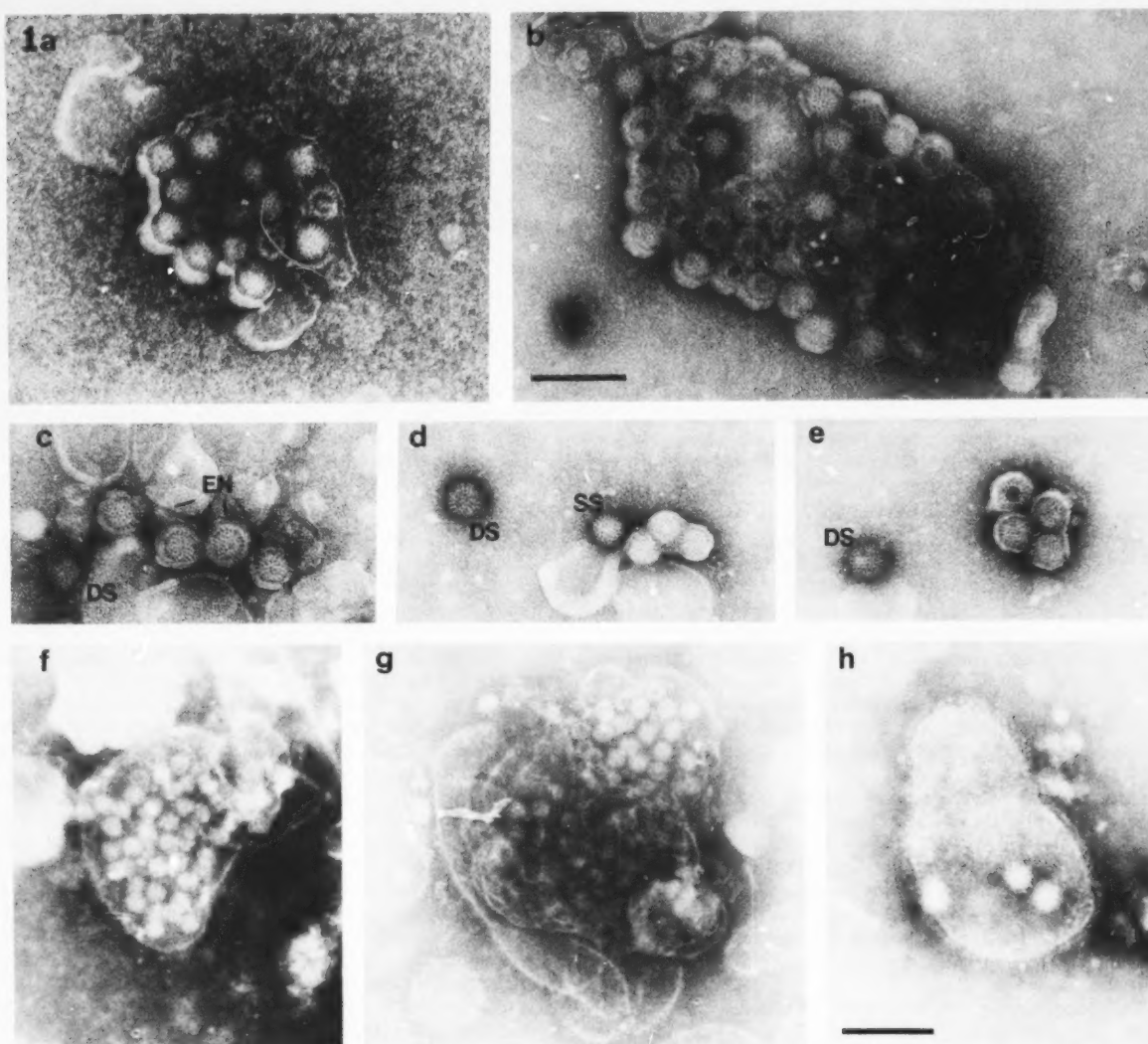


FIG. 1. Membrane-associated virus particles in stool specimens. (a and b) Viral-packet complexes of rotavirus in one specimen. (c through e) Individual enveloped particles (EN), double-shelled particles (DS), single-shelled particles (SS), and small groups of membrane-associated particles in the same specimen. Bar for panels a through e, 0.2 μ m. (f and g) Parvovirus-like viral packets observed in another stool specimen. (h) Three Norwalk virus particles associated with a fuzzy membranous element in another stool specimen. Bar for panels f through h, 0.1 μ m.

Membrane-associated viral complexes were often irregularly stained and presented a highly varied appearance. Many viral complexes undoubtedly remained embedded among masses of cellular debris and were not detected. Many individual enveloped virions must have also remained undetected in cases in which the outer membrane completely obscured the distinctive rotavirus structure.

EM photographic plates of stool specimen viruses observed during other investigations and kept for reference were reviewed for the presence of membrane-associated virions. Plates of five other rotavirus-containing stool specimens revealed membrane-associated virions in one stool specimen. Interestingly, a review of EM plates of stool specimens containing other virus types revealed the presence of membrane-associated parvovirus-like particles in

three stool specimens. Two viral-packet complexes observed in one of these stool specimens appeared to contain ca. 40 virions each (Fig. 1f and g). A micrograph of Norwalk virus particles (Fig. 1h) in a stool specimen confirmed to be positive by a Norwalk virus radioimmunoassay (5) suggested that the membrane-associated state might also occur with that virus.

Figure 2a through c shows rotavirus packets observed at different passages in the cell culture fluids. These packets were electron microscopically identical to those seen in the crude stool specimen. In their longest dimension, these three packets measured 0.6 μ m (a), 1.4 μ m (b), and 0.4 μ m (c) and appeared to contain ca. 30, 100, and 13 virions, respectively. The virions appeared to be complete double-shelled particles, as in the stool specimen. Individual membrane-

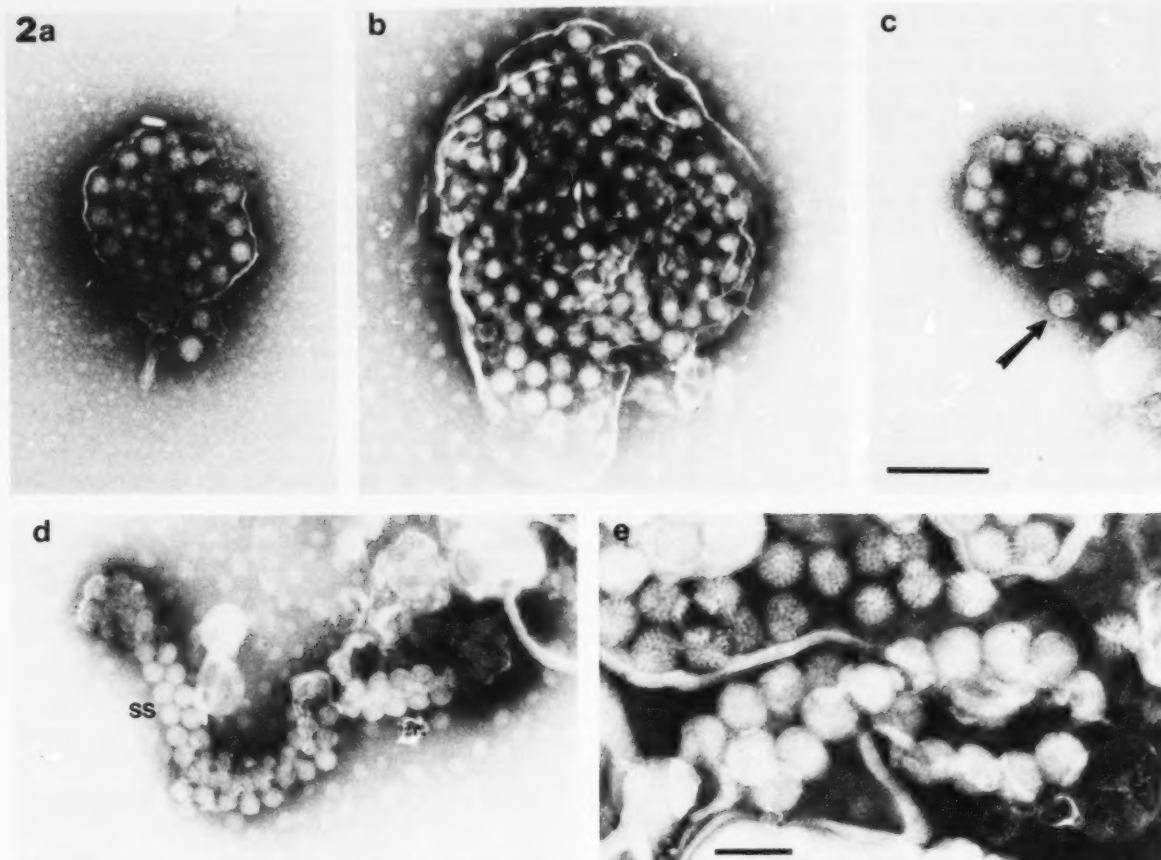


FIG. 2. Membrane-associated rotavirus particles in cell culture fluids. (a) Viral packet from passage 3 in RD cells. (b) Large packet containing ca. 100 virions from passage 5. (c) Packet from passage 19. Note the individual enveloped particle (arrow). (d) Viral chain consisting of both single-shelled (SS) and enveloped (EN) particles. Bar for panels a through d, 0.3 μ m. (e) Single chain consisting of enveloped particles. Bar, 0.1 μ m.

enveloped virions were also present (c). The presence of the membrane-associated viral complexes through passage 19 demonstrated that they were not residual complexes from the inoculum and suggested that such membrane-associated virions naturally occur with *in vivo* or *in vitro* replication.

Of unusual appearance were the "viral chains" observed in the cell culture fluids (Fig. 2d and e). Both enveloped and nonenveloped virions were observed in these chains. They were only infrequently observed, and their significance is not known, but they did not appear to be an artifact of the EM procedure.

Virus particles contained within membranous sacs have previously been observed in cell culture harvests of bovine rotavirus (1). Similar membrane-localized virus formations have more recently been observed in cell culture lysates after inoculation with human rotavirus (11). These earlier reports of membranous formations of viruses appear to correspond to the membrane-associated rotavirus complexes observed here in both cell culture fluids and stool material. Such complexes are apparently formed during the process of virus maturation in the host cells. This maturation process has been described for rotavirus in a number of thin section studies (2, 9, 11, 13, 16, 17). Although individual interpretation may vary on the exact details of this process,

virus maturation clearly involves the interaction of the virions with the membrane of the rough endoplasmic reticulum. Immature virus particles pass through this membrane, accumulating in the cisternae of the rough endoplasmic reticulum, and in the process develop into mature double-shelled virions. The observations reported in this study indicate that many of these virions can be shed in the stools of infected individuals while still attached to, or entrapped within, membrane complexes of the rough endoplasmic reticulum. As most of these complexes were observed to contain mature double-shelled virions, these viral packets must be considered to be potentially infectious.

Although recent results with simian rotavirus SA11 indicate that chlorine and chlorine dioxide are effective in inactivating both individual and cell-associated virions (4), other data suggest that stool-shed human rotaviruses may be more resistant to disinfection than SA11 (7). Clearly, further studies are required to determine the disinfection characteristics of such naturally occurring rotaviruses. Such studies should consider the membrane-associated complexes described in this report.

The detection in several stool specimens examined in this laboratory of membrane-associated complexes of parvovirus-like particles suggests that this phenomenon is not re-

stricted to rotavirus and may apply to other stool-shed viruses. A similar observation of membrane-associated parvovirus-like particles has been reported in England (6).

Membrane-associated viral complexes can be difficult to visualize. However, it is clear that these complexes do occur in stools and can be expected to be shed into the water environment. Water disinfection procedures must adequately address the likely presence and increased resistance of such viral aggregates if treated water from sewage-contaminated sources is to be considered free of viral contamination.

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